American Journal of Hematology 60:158–160 (1999)

Heterogeneity of Clonal Development in Chronic Myeloproliferative Disorders

Anna Maria Ferraris,[1]* Rosa Mangerini,[1] Omar Racchi,[1] Davide Rapezzi,[1] Michela Rolfo,[1] Salvatore Casciaro,[2] and Gian Franco Gaetani[1]

[1]Dipartimento di Oncologia Clinica e Sperimentale, Universitá di Genova and Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy
[2]Divisione di Medicina, Ospedale San Martino, Genova, Italy

Recent reports have suggested a previously unexpected variability in the expression of the dominant neoplastic clone in myeloproliferative disorders (MPD). We evaluated 49 female patients with MPD and informative at the X-linked androgen receptor (AR) locus to establish the X chromosome inactivation pattern of hemopoietic cells. Whereas in chronic myelogenous leukemia (CML) the granulocytes (PMN) were uniformly of monoclonal origin, a striking heterogeneity of clonal development was found in PMN from patients with other MPD, with up to 50% of them expressing a polyclonal pattern of X inactivation. Am. J. Hematol. 60:158–160, 1999. © 1999 Wiley-Liss, Inc.

Key words: clonality; X inactivation; myeloproliferative

INTRODUCTION

The myeloproliferative disorders (MPD) are a diverse group of hemopoietic diseases characterized by abnormal proliferation of a subset of marrow elements. Previous studies have shown that they are clonal diseases that arise in a multipotent hemopoietic stem cell [1,2]. Recent findings however have suggested a variability in the dominance of the neoplastic clone in some MPD patients [3].

X chromosome-linked markers have been successfully used to study the origin and development of human neoplasms [4]. Following inactivation of one X chromosome in somatic cells of females, women heterozygous for polymorphic X-linked genes have a mosaic of cells expressing one or the other allele in their normal tissues, whereas a tumor arising from a single cell will show a homogeneous phenotype.

Taking advantage of a newly described clonality assay based on the methylation of *Hpa*II and *Hha*I sites within a highly polymorphic trinucleotide repeat in the coding region of the X-linked human androgen receptor (AR) gene [5], we sought to determine the expression of X chromosome alleles in hemopoietic cell populations from a group of patients with MPD.

PATIENTS AND METHODS

X chromosome inactivation pattern was assessed in 49 female patients with MPD; informed consent was obtained from all subjects. According to the criteria of the Polycythemia Vera Study Group [6,7], 20 had chronic myelogenous leukemia (CML), 13 had essential thrombocythemia (ET), 11 had polycythemia vera (PV), and 5 had idiopathic myelofibrosis (IMF). All patients with CML were Ph positive and in chronic phase. The mean age of the patients was 61 years (range 21–92); 35 were analyzed at the time of diagnosis, and 14 several years thereafter (mean 7.3 years, range 2–22). Eight subjects (2 CML, 2 PV, 3 ET, and 1 IMF) were studied at various intervals during the course of their disease (mean 6.1 years, range 2–10).

Granulocytes (PMN) and mononuclear cells (FH) were purified from peripheral blood according to standard methods [2]. Monocytes were removed from the FH fraction by adhesion to plastic culture dishes. B and T lymphocytes were further isolated with magnetic immunobeads coated with the appropriate antibody (Dynal, Norway). The degree of contamination of each cell frac-

Contract grant sponsor: Ricerca Finalizzata Ministero Sanitá 1996; Contract grant number: RF'96.286/ICS070.1; Contract grant sponsor: MURST 1996–1997 and 1997–1998; Contract grant sponsor: P.F. Biotecnologie (CNR Target Project on Biotechnology).

*Correspondence to: Dr. Anna Maria Ferraris, Ematologia Oncologica, IST, Largo Rosanna Benzi, 10, 16132 Genova, Italy.

Received for publication 25 February 1998; Accepted 12 August 1998

TABLE I. X Chromosome Inactivation Patterns in Hemopoietic Cells from MPD Patients*

	PMN		FH		B		T	
	C	NC	C	NC	C	NC	C	NC
CML (20)[a]	20	0	—	—	1	3	1	1
PV (11)	7	4	2	8	0	1	0	3
ET (13)	6	7	1	10	0	2	0	7
IMF (5)	3	2	2	1	0	1	—	—

*MPD, myeloproliferative disorder; PMN, granulocytes; FH, mononuclear cells; B, B lymphocytes; T, T lymphocytes; C, monoclonal; NC, polyclonal; CML, chronic myelogenous leukemia; PV, polycythemia vera; ET, essential thrombocythemia; IMF, idiopathic myelofibrosis.
[a]Total number of patients tested.

tion was less than 3% by direct examination of cytocentrifuge slides stained with May-Grünwald-Giemsa.

The recent identification [5] of consistently methylated differential sites between the active and inactive X chromosome, closely linked to the multiallelic CAG short tandem repeat of the first exon of the human AR gene, allows a more accurate determination of X chromosome inactivation pattern than previous methods, and has greatly contributed to the reliability and reproducibility of research in the field of X inactivation analysis. In the present study, assessment of clonality at the AR locus was then performed by polymerase chain reaction (PCR) amplification according to a modification of the technique of Allen et al. [5], as described elsewhere [8].

RESULTS

In 210 control women, the rate of heterozygosity at the AR locus was 88%. X chromosome inactivation pattern was determined in hemopoietic cells (PMN and FH) from 119 normal females informative at the AR locus. Identification of the minor component was unequivocal when it constituted at least 5% of the total. Visual assessment of relative hybridization intensities was confirmed by densitometric analysis of autoradiogram bands. The allelic ratio after *Hpa*II digestion was consistent with random X inactivation [8].

From the 56 patients with MPD originally tested, 49 (87.5%) were informative at the AR locus and therefore suitable for X chromosome inactivation analysis.

In all CML cases, PMN showed a monoclonal pattern of X inactivation; B and T lymphocytes were polyclonal, except for one case (Table I). The other MPD (PV, ET, IMF) proved to be heterogeneous: PMN were monoclonal in 64% of the patients with PV, 46% with ET, and 60% with IMF. FH cells were monoclonal in 20% of PV patients, 9% of ET patients, and 67% of IMF patients. B lymphocytes were available from 2 ET cases, 1 PV case and 1 IMF case, and always expressed a polyclonal pattern of X inactivation. T lymphocytes were isolated in 7 ET cases and 3 PV cases and were polyclonal as well (Table I). Figure 1 illustrates a representative example of

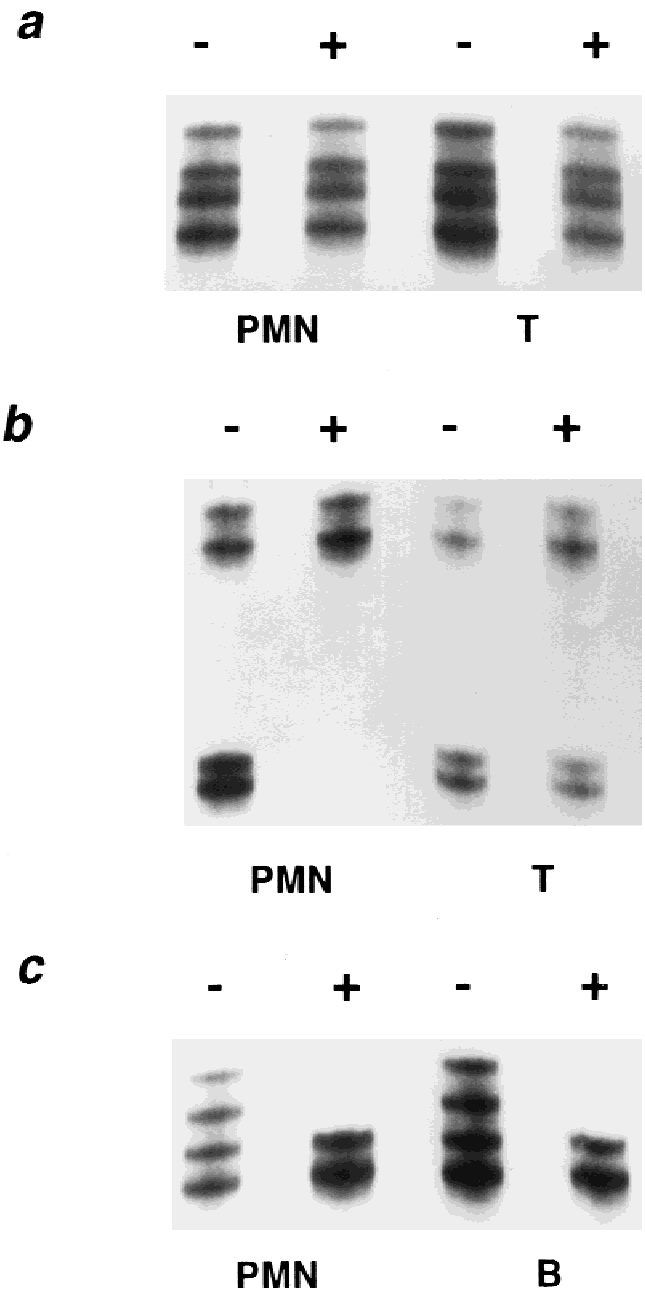

Fig. 1. Clonal analysis of three patients with MPD with the AR polymorphic marker. DNA isolated from granulocytes (PMN), T lymphocytes (T) and B lymphocytes (B) was predigested with (lanes marked +) and without (lanes marked −) the methylation sensitive enzyme *Hpa*II. Each allele is represented by two bands. a. In this patient with ET, PMN and T cells express a polyclonal pattern of X inactivation. b. In another ET case, absence of the lower allele in the PMN after *Hpa*II digestion (lane +) indicates a monoclonal origin. c. In this CML patient, both PMN and B cells show a monoclonal pattern of X inactivation.

X inactivation analysis in hemopoietic cells from one patient with CML and two with ET.

Our results are in agreement with recent reports on smaller numbers of MPD patients as to the variability in

the dominance of the neoplastic clone in PMN; studies on lymphoid cells have been much more sporadic and their findings cannot be generalized. In fact, whereas we were able to obtain purified PMN from all subjects, the same has not been feasible for the other cell populations (Table I). In the CML cases, this has been primarily due to the well known quantitative and qualitative abnormalities of myeloid cells in this disease, making separation procedures for lymphoid cells extremely cumbersome and inaccurate. Moreover, although attempts to obtain as many purified cell populations as possible were performed in all subjects studied prospectively, several samples had been collected and stored in the past for previous clonality studies, and the patients were no longer available for further separation procedures.

Duration of the disease did not affect the expression of clonality. In the eight cases tested repeatedly during the course of the disease, the same pattern of X chromosome inactivation found at diagnosis was confirmed in all subsequent analyses, up to 10 years later. Five subjects (2 CML, 1 PV, 1 ET, and 1 IMF) maintained a clonal hematopoiesis and three others (1 PV and 2 ET) were persistently polyclonal.

In order to disclose a possible correlation between clinical manifestation(s) of the disease and the heterogeneity of clonal development found in hemopoietic cells from our patients with PV, ET, and IMF (Table I), we analyzed several clinical parameters, namely level of hemoglobin, white cells and platelets counts, splenomegaly, and the need for myelosuppressive treatment. None of these characteristics correlates with clonality. Specifically, ET patients had a platelet count at diagnosis within the same range (700–1,000 $\times$ 10^9/L), whether their PMN were monoclonal or polyclonal. Events such as hemorrhage or thrombosis were very rare in our group and therefore these parameters could not be evaluated properly.

DISCUSSION

The classical notion [1,9] of MPD as the result of a clonal development from a multipotent stem cell has recently been challenged by the evidence of variable clonal involvement of hematopoietic cell populations in some patients with these diseases [3]. Our finding of polyclonal PMN in approximately half of the patients fully validates the suggestion of some form of heterogeneity of origin of PV, ET, and IMF. Analysis of the 20 CML cases showed a completely different picture, that is a consistently clonal pattern of X inactivation in the myeloid cells (Table I). This finding is in agreement with previously reported clonality studies in CML [1,9] and strongly suggests a pathogenetic process different from the other MPD. The difference in stringency of diagnostic criteria between CML and the other MPD provides a simple but plausible explanation for this heterogeneity. Furthermore, although a wealth of clinical and biomolecular studies on CML have firmly established this disease as a truly neoplastic one, with a multistep pathogenesis of consecutive malignant events, high-quality investigations on the other MPD have been more scattered.

Molecular heterogeneity in our ET, PV, and IMF patients did not correlate with usual clinical parameters; however, in the cell populations examined the consistency of the pattern of X chromosome inactivation hints to more subtle differences yet to be uncovered, potentially of great relevance as prognostic indicators of new therapeutic strategies.

REFERENCES

1. Fialkow PJ. Cell lineages in hematopoietic neoplasia studied with glucose-6-phosphate dehydrogenase cell markers. J Cell Physiol (suppl) 1982;1:37.
2. Gaetani GF, Ferraris AM, Galiano S, Giuntini P, Canepa L, D'Urso M. Primary thrombocythemia: clonal origin of platelets, erythrocytes and granulocytes in a $Gd^B/Gd^{Mediterranean}$ subject. Blood 1982;59:76.
3. El Kassar N, Hetet G, Li Y, Brière J, Grandchamp B. Clonal analysis of haemopoietic cells in essential thrombocythaemia. Br J Haematol 1995;90:131.
4. Wainscoat JS, Fey MF. Assessment of clonality in human tumors: A review. Cancer Res 1990;50:1355.
5. Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont JW. Methylation of Hpa II and Hha I sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X-chromosome inactivation. Am J Hum Genet 1992;51:1229.
6. Berlin NI. Diagnosis and classification of the polycythemias. Semin Hematol 1975;12:339.
7. Laszlo J. Myeloproliferative disorders (MPD): myelofibrosis, myelosclerosis, extramedullary hematopoiesis, undifferentiated MPD, and hemorrhagic thrombocythemia. Semin Hematol 1975;12:409.
8. Ferraris AM, Mangerini R, Gaetani GF, Romei C, Pinchera A, Pacini F. Polyclonal origin of medullary carcinoma of the thyroid in multiple endocrine neoplasia type 2. Hum Genet 1997;99:202.
9. Gilliland DG, Blanchard KL, Bunn HF. Clonality in acquired hematologic disorders. Ann Rev Med 1991;42:491.